PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N

A2

(11) International Publication Number: WO 00/40693

(43) International Publication Date: 13 July 2000 (13.07.00)

(21) International Application Number: PCT/US00/00264

(22) International Filing Date: 6 January 2000 (06.01.00)

(30) Priority Data:
60/114,995 6 January 1999 (06.01.99) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/114,995 (CIP)
Filed on 6 January 1999 (06.01.99)

(71) Applicant *(for all designated States except US)*: ATLANTIC BIOPHARMACEUTICALS, INC. [US/US]; 50 Church Street, Cambridge, MA 02138 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LINDSAY, Stace [US/US]; 8 Cypress Street, Cambridge, MA 02140 (US). MULROY, Robert [US/US]; 19 Hilliard Street, Cambridge, MA 02138 (US). SEMENIUK, Daniel [US/US]; 1860 Thousand Oaks Lane, Lawrenceville, GA 30043 (US).

(74) Agent: CLARK, Paul, T.; Clark & Elbing LLP, 176 Federal Street, Boston, MA 02110-2214 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: EXPRESSION OF SECRETED HUMAN ALPHA-FETOPROTEIN IN TRANSGENIC ANIMALS

(57) Abstract

The invention features a process of expressing secreted recombinant human alpha-fetoprotein (rHuAFP) in the milk or urine of transgenic mammals.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

EXPRESSION OF SECRETED HUMAN ALPHA-FETOPROTEIN IN TRANSGENIC ANIMALS

Background of the Invention

This invention relates to the expression and secretion of recombinant protein in transgenic animals.

Alpha-fetoprotein (AFP) is a 70 kDa glycoprotein produced by the yolk sac and fetal liver. AFP is present in fetal serum at milligram levels, and, at birth, declines to the nanogram levels normally found in adult serum: increased levels of AFP in adult serum are indicative of a yolk sac tumor, a hepatoma, or of liver regeneration. The role of AFP during fetal development is not known, although it has been suggested that AFP may protect a gestating fetus from a maternal immune attack or from the effects of maternal estrogen.

In vitro and *in vivo* experiments have shown that AFP has both cell growth-stimulatory and -inhibitory activities, depending upon the target cell, the relative concentration of AFP, and the presence of other cytokines and growth factors. For example, AFP can inhibit the growth of many types of tumor cells, and, in particular, inhibits estrogen-stimulated cell growth. Conversely, AFP stimulates the growth of normal embryonal fibroblasts. AFP has also been shown to have both immunosuppressive and immunoproliferative effects. In order to exploit the various biological properties of AFP, it will be necessary to obtain sufficient quantities of this molecule in an efficient and cost-effective manner.

Summary of the Invention

In a first aspect, the invention features a substantially pure nucleic acid molecule comprising: (i) a nucleic acid sequence encoding recombinant

human alpha-fetoprotein (rHuAFP), (ii) a milk-specific promoter, the promoter being operably linked to the rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of rHuAFP by milk-producing cells into the milk of a mammal.

In a second aspect, the invention features a substantially pure nucleic acid molecule comprising: (i) a nucleic acid sequence encoding recombinant human alpha-fetoprotein (rHuAFP), (ii) a urine-specific promoter, the promoter being operably linked to the rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of rHuAFP by urine-producing cells into the urine of a mammal.

In a third aspect, the invention features a non-human transgenic mammal that expresses recombinant human alpha-fetoprotein (rHuAFP) in its milk, wherein milk-producing cells of the mammal contain a transgene that comprises: (i) a nucleic acid sequence encoding rHuAFP, (ii) a milk-specific promoter, the promoter being operably linked to the rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of rHuAFP by milk-producing cells into the milk of a mammal.

In a fourth aspect, the invention features a non-human transgenic mammal that expresses recombinant human alpha-fetoprotein (rHuAFP) in its urine, wherein urine-producing cells of the mammal contain a transgene that comprises: (i) a nucleic acid sequence encoding rHuAFP, (ii) a urine-specific promoter, the promoter being operably linked to the rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of rHuAFP by urine-producing cells into the urine of an animal.

In preferred embodiments of the third and fourth aspects of the invention, the mammal may be a goat, a cow, a sheep, or a pig.

In a fifth aspect, the invention features a non-human mammal's milk comprising recombinant human alpha-fetoprotein (rHuAFP). In a preferred embodiment of the fifth aspect of the invention, the rHuAFP is soluble and is produced by a non-human transgenic mammal whose milk-producing cells express a transgene that comprises: (i) a nucleic acid sequence encoding rHuAFP, (ii) a milk-specific promoter, the promoter being operably linked to the rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of rHuAFP by the milk-producing cells into the milk of the mammal.

In a sixth aspect, the invention features a non-human mammal's urine comprising recombinant human alpha-fetoprotein (rHuAFP). In a preferred embodiment of the sixth aspect of the invention, the rHuAFP is soluble and is produced by a non-human transgenic mammal whose urine-producing cells express a transgene that comprises: (i) a nucleic acid sequence encoding rHuAFP, (ii) a urine-specific promoter, the promoter being operably linked to the rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of rHuAFP by the urine-producing cells into the urine of the mammal.

In a seventh aspect, the invention features a method of producing recombinant human alpha-fetoprotein (rHuAFP) that is secreted in the milk of a mammal, comprising the steps of: (a) providing a cell transfected with a transgene that comprises: (i) a nucleic acid sequence encoding rHuAFP, (ii) a milk-specific promoter, the promoter being operably linked to the rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of rHuAFP by a milk-producing cell, wherein the milk-producing cell is derived from said transfected cell; (b) growing the cell to produce a mammal comprising milk-producing cells that express and secrete rHuAFP into milk; and collecting milk containing rHuAFP from the mammal.

In one preferred embodiment, the rHuAFP is purified from the milk.

In an eighth aspect, the invention features a method of producing recombinant human alpha-fetoprotein (rHuAFP) that is secreted in the urine of a mammal, comprising the steps of: (a) providing a cell transfected with a transgene that comprises: (i) a nucleic acid sequence encoding rHuAFP, (ii) a urine-specific promoter, the promoter being operably linked to the rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of rHuAFP by a urine-producing cell, wherein the urine-producing cell is derived from the transfected cell; (b) growing the cell to produce a mammal comprising urine-producing cells that express and secrete the rHuAFP into the urine; and (c) collecting urine containing rHuAFP from the mammal. In one preferred embodiment, rHuAFP is purified from the urine.

In a ninth aspect, the invention features a method of treating a patient in need of recombinant human alpha-fetoprotein (rHuAFP), including administering to the patient a therapeutically-effective amount of non-human mammal's milk containing recombinant human alpha-fetoprotein (rHuAFP).

In a preferred embodiment of the ninth aspect of the invention, the rHuAFP is produced by a non-human transgenic mammal whose milk-producing cells contain a transgene that comprises: (i) a nucleic acid sequence encoding rHuAFP, (ii) a milk-specific promoter, the promoter being operably linked to the rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of rHuAFP by milk-producing cells into the milk of the mammal.

In a tenth aspect, the invention features a method of treating a patient in need of recombinant human alpha-fetoprotein (rHuAFP), comprising administering to the patient a therapeutically-effective amount of recombinant human alpha-fetoprotein (rHuAFP) purified from a non-human mammal's urine.

In preferred embodiments of the tenth aspect of the invention, the rHuAFP is produced by a non-human transgenic mammal whose urine-producing cells contain a transgene that comprises: (i) a nucleic acid sequence encoding rHuAFP, (ii) a urine-specific promoter, the promoter being operably linked to the rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of rHuAFP by urine-producing cells into the urine of the mammal.

In various preferred embodiments of the ninth and tenth aspects of the invention, the method may be used for the treatment of cancer, for suppressing the immune system, or for inducing proliferation of bone marrow cells in a patient in need thereof.

By "human alpha-fetoprotein" or "HuAFP" or "rHuAFP" is meant a polypeptide having substantially the same amino acid sequence as the mature alpha-fetoprotein (amino acids 20-609) set forth in Genbank Accession No. J00077 and encoded by the cDNA sequence set forth in Genbank Accession No. J00077 and reported in Morinaga *et al.* (*Proc. Natl. Acad. Sci.* USA 80:4604-4608, 1983).

By "human alpha-fetoprotein precursor" is meant a polypeptide having substantially the same amino acid sequence as amino acids 1-609 set forth in Genbank Accession No. J00077.

By "having substantially the same amino acid sequence" is meant a polypeptide that exhibits at least 80% identity with a naturally-occurring HuAFP amino acid sequence, typically at least about 85% identity with a naturally-occurring human HuAFP sequence, more typically at least about 90% identity, usually at least about 95% identity, and more usually at least about 97% identity with a naturally-occurring HuAFP sequence. The length of comparison sequences will generally be at least 16 amino acids, usually at least 20 amino acids, more usually at least 25 amino acids, typically at least 30

amino acids, and preferably more than 35 amino acids.

Sequence identity is typically measured using sequence analysis software with the default parameters specified therein, such as the introduction of gaps to achieve an optimal alignment (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705).

By "milk-producing cell" is meant a mammary epithelial cell that secretes milk.

By "urine-producing cell" is meant a bladder epithelial cell that secretes urine.

By "promoter" is meant a minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell type-specific, tissue-specific, temporal-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' or intron sequence regions of the native gene.

By "milk-specific promoter" is meant a promoter that naturally directs expression of a gene that is expressed in mammary epithelial cells, for example, the native promoter associated with the genes encoding whey acidic protein (WAP), alpha S1-casein, alpha S2-casein, beta-casein, kappa-casein, beta-lactoglobulin, and alpha-lactalbumin.

By "urine-specific promoter" is meant a promoter that naturally directs expression of a gene that is expressed in bladder epithelial cells, for example, the uroplakin II promoter.

By "recombinant HuAFP" or "rHuAFP" is meant human alpha-fetoprotein encoded by an artificially-constructed nucleic acid.

By "exogenous," as used herein in reference to a gene or a polypeptide, is meant a gene or polypeptide that is not normally present in an

animal. For example, rHuAFP is exogenous to a goat.

By "purified" is meant that rHuAFP secreted into milk or urine is partially or completely separated from other components (*e.g.*, proteins, lipids, and water) naturally found in milk or urine, thus increasing the effective concentration of rHuAFP relative to unpurified rHuAFP found in milk or urine.

By "substantially pure nucleic acid" is meant nucleic acid that is free of the genes which, in the naturally-occurring genome of the organism from which the nucleic acid of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (*e.g.*, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA that is part of a hybrid gene containing a nucleotide sequence not native to the gene or encoding additional polypeptide sequence, as well as the corresponding mRNA.

By "transformation" or "transfection" or "transduction" is meant any method for introducing foreign molecules into a cell. Lipofection, DEAE-dextran-mediated transfection, microinjection, protoplast fusion, calcium phosphate precipitation, transduction (*e.g.*, bacteriophage, adenoviral retroviral, or other viral delivery), electroporation, and biolistic transformation are just a few of the methods known to those skilled in the art which may be used.

By "transformed cell" or "transfected cell," or "transduced cell," is meant a cell (or a descendent of a cell) into which a DNA molecule encoding rHuAFP has been introduced, by means of recombinant DNA techniques. The DNA molecule may be stably incorporated into the host chromosome, or may be maintained episomally.

By "operably linked" is meant that a gene and one or more

regulatory sequences are connected in such a way as to permit gene expression when the appropriate molecules (*e.g.*, transcriptional activator proteins) are bound to the regulatory sequences.

By "expression vector" is meant a genetically engineered plasmid or virus, derived from, for example, a bacteriophage, adenovirus, retrovirus, poxvirus, herpesvirus, or artificial chromosome, that is used to transfer an rHuAFP coding sequence, operably linked to a promoter, into a host cell, such that the encoded rHuAFP is expressed within the host cell.

By "embryonal cell" is meant a cell that is capable of being a progenitor to all the somatic and germ-line cells of an organism. Exemplary embryonal cells are embryonic stem cells (ES cells) and fertilized oocytes. Preferably, the embryonal cells of the invention are mammalian embryonal cells.

By "transgene" is meant any piece of nucleic acid that is inserted by artifice into a cell, or an ancestor thereof, and becomes part of the genome of the animal which develops from that cell. Such a transgene may include a gene which is partly or entirely exogenous (*i.e.*, foreign) to the transgenic animal, or may represent a gene having identity to an endogenous gene of the animal.

By "transgenic" is meant any cell that includes a nucleic acid sequence that has been inserted by artifice into a cell, or an ancestor thereof, and becomes part of the genome of the animal which develops from that cell. Preferably, the transgenic animals are transgenic mammals (*e.g.*, goats, sheep, cows, and pigs). Preferably the nucleic acid (transgene) is inserted by artifice into the nuclear genome (*i.e.*, a chromosome), although the transgene may also be episomally maintained (*e.g.*, carried on a vector that contains an origin of replication such as the Epstein-Barr Virus oriP).

By a "leader sequence" or a "signal sequence" is meant a nucleic acid sequence that encodes a protein secretory signal, and, when operably

linked to a downstream nucleic acid molecule encoding rHuAFP, directs rHuAFP secretion. The leader sequence may be the native rHuAFP leader, an artificially-derived leader, or may obtained from the same gene as the promoter used to direct transcription of the rHuAFP coding sequence, or from another protein that is normally secreted from a cell.

By "human alpha-fetoprotein secretory signal" or "human alpha-fetoprotein signal peptide" or "human alpha-fetoprotein leader" or "human alpha-fetoprotein signal sequence" is meant a polypeptide having substantially the same amino acid sequence amino acids 1-19 set forth in Genbank Accession No. J00077. The protein secretory signal is cleaved from HuAFP during protein maturation and extracellular secretion.

By "therapeutically-effective amount" is meant an amount of recombinant human alpha-fetoprotein or fragment thereof that when administered to a patient inhibits or stimulates a biological activity modulated by human alpha-fetoprotein. Such biological activities include inhibiting the proliferation of a neoplasm or an autoreactive immune cell, or stimulating proliferation of a cell (*e.g.*, a bone marrow cell). The therapeutically-effective amount may vary depending upon a number of factors, including medical indication, the length of time of administration and the route of administration. For example, rHuAFP can be administered systemically in the range of 0.1 ng - 10g/kg body weight, preferably in the range of 1 ng - 1g/kg body weight, more preferably in the range of 10 ng - 100mg/kg body weight, and most preferably in the range of 1μg-10 mg/kg body weight.

Brief Description of the Drawings

Figure 1 is a diagram showing the structure of a goat beta-casein/rHuAFP transgene for expression and secretion of rHuAFP into milk.

Figure 2 is a diagram showing the genomic organization of the

human AFP gene and the two overlapping lambda (λ) fragments.

Figure 3 is a diagram showing the design of a vector including the 5' subclone of the human AFP gene and an expression construct.

Figure 4 is a diagram showing the design of a vector including the 3' subclone of the human AFP gene and an expression construct.

Figure 5 is a diagram showing a strategy for linking the 5' and 3' AFP gene fragments and inserting the entire human AFP genomic fragment into the GTC beta-casein expression vector.

Detailed Description of the Invention

The present invention features a process for expressing secreted recombinant human alpha-fetoprotein (rHuAFP) in transgenic mammals, particularly ruminants (*e.g.*, cattle, sheep, and goats). The transgene that directs expression of secreted rHuAFP contains the human AFP coding region fused downstream of a nucleic acid containing a transcriptional promoter. Between the promoter and the protein coding region is a leader sequence encoding a protein secretory signal. Depending upon the promoter and secretory signal employed, the transgene-encoded rHuAFP is secreted into the milk or urine of the transgenic animal. Additional nucleic acid elements, such as transcriptional enhancers, transcriptional and translational terminator sequences, 3' untranslated regions that enhance mRNA stability, and introns that enhance expression may also be included in the transgenic construct.

Production of rHuAFP by secretion into milk or urine facilitates its purification and obviates removal of blood products and culture medium additives, some of which may be toxic, carcinogenic, or infectious. Moreover, milk containing rHuAFP may be directly consumed by humans or other mammals. Expression of rHuAFP in urine allows the use of both male and female animals for rHuAFP production. In addition, rHuAFP is produced as

soon as the animals begin to produce urine. Finally, purification of rHuAFP from urine is relatively straightforward, as urine normally contains a low protein content.

Transgene constructs

Useful promoters for the expression of a rHuAFP transgene in mammary tissue include promoters that naturally drive the expression of mammary-specific proteins, such as milk proteins, although any promoter that permits secretion of the transgene product into milk may be used. These include, *e.g.*, the promoters that naturally direct expression of whey acidic protein (WAP), alpha S1-casein, alpha S2-casein, beta-casein, kappa-casein, beta-lactoglobulin, and alpha-lactalbumin (see, *e.g.*, Drohan *et al.*, U.S.P.N. 5,589,604; Meade *et al.* U.S. Patent No. 4, 873,316; and Karatzas *et al.*, U.S. patent No. 5,780,009).

A useful promoter for the expression of an rHuAFP transgene in urinary tissue is the uroplakin promoter (Kerr *et al.*, *Nat. Biotechnol.* 16:75-79, 1998), although any promoter that permits secretion of the transgene product into urine may be used.

The transgene construct preferably includes a leader sequence downstream from the promoter. The leader sequence is a nucleic acid sequence that encodes a protein secretory signal, and, when operably linked to a downstream nucleic acid molecule encoding rHuAFP, directs rHuAFP secretion. The leader sequence may be obtained from the same gene as the promoter used to direct transcription of the nucleic acid molecule encoding rHuAFP (for example, a gene that encodes a milk-specific protein). Alternatively, a leader sequence encoding the native rHuAFP protein secretory signal (amino acids 1-19 of Genbank Accession No. J00077) may be employed: nucleotides 45-101 of Genbank Accession No. J00077 encode the native

HuAFP protein secretory signal. Other options include use of a leader sequence that encodes a protein secretory signal from any other protein that is normally secreted from a cell, an artificial leader sequence that encodes an artificial protein secretory signal, or a hybrid leader sequence (*e.g.*, a fusion of the goat beta-casein and HuAFP leader sequences).

In addition, the transgene construct preferably includes a transcription termination site, a signal for polyadenylation of the transcribed mRNA, and a translation termination signal. The transgene may also encode any 3' untranslated region (UTR), which increases stability of the rHuAFP mRNA, for example, a 3' UTR from the bovine growth hormone gene, a milk protein gene, or a globin gene.

The transgene construct may also include a transcriptional enhancer upstream or downstream from the transcribed region of the transgene, such as an enhancer from a viral (*e.g.*, SV40) or mammalian (*e.g.*, casein) gene.

The transgene construct may further include an intron that increases the level of expression of the transgene. The intron may be placed between the transcription initiation site and the translational start codon, 3' of the translational stop codon, or within the coding region of the transgene. The intron should include a 5' splice site (*i.e.*, a donor site), a 3' splice site (*i.e.*, an acceptor site), and preferably, at least 100 nucleotides between the two sites. Any intron that is known in the art to increase expression of a transgene (*e.g.*, an intron from a ruminant casein gene) may be used.

The transgene construct may include genomic or cDNA that expresses HuAFP or a fragment thereof. Exemplary fragments of HuAFP are described in Murgita , WO 96/2287. In addition, the transgene may be engineered to express a rHuAFP molecule that is non-glycosylated. This is accomplished by mutating the codon encoding the single N-linked glycosylation site of the AFP molecule using standard methods known in the

art.

The rHuAFP transgene may be carried within a circular plasmid, a cosmid vector, or other vector, such as a vector derived from a virus. The vector may contain additional sequences that facilitate its propagation in prokaryotic and eukaryotic cells, for example, drug-selectable markers (*e.g.*, for ampicillin resistance in *E. coli*, or G-418 resistance in mammalian cells) and origins of replication (*e.g.*, colE1 for replication in prokaryotic cells, and oriP for replication in mammalian cells).

Generation of Transgenic Animals

Transgenic constructs are usually introduced into cells by microinjection (Ogata *et al.*, U.S. Patent No. 4,873,292). A microinjected embryo is then transferred to an appropriate female resulting in the birth of a transgenic or chimeric animal, depending upon the stage of development of the embryo when the transgene integrated. Chimeric animals can be bred to form true germline transgenic animals.

In some methods of transgenesis, transgenes are introduced into the pronuclei of fertilized oocytes. For some animals, such as mice, fertilization is performed *in vivo* and fertilized ova are surgically removed. In other animals, the ova can be removed from live, or from newly-dead (*e.g.*, slaughterhouse) animals and fertilized *in vitro*.

Alternatively, transgenes can be introduced into embryonic stem cells (ES cells). Transgenes can be introduced into such cells by electroporation, microinjection, or any other techniques used for the transfection of cells which are known to the skilled artisan. Transformed cells are combined with blastocysts from the animal from which they originate. The transformed cells colonize the embryo, and in some embryos these cells form the germline of the resulting chimeric animal (Jaenisch, R., *Science* 240:

1468-1474, 1988).

ES cells containing an rHuAFP transgene may also be used as a source of nuclei for transplantation into an enucleated fertilized oocyte, thus giving rise to a transgenic animal. More generally, any diploid cell derived from embryonic, fetal, or adult tissue and containing an rHuAFP transgene may be introduced into an enucleated unfertilized egg. The cloned embryo is implanted and gestated within an appropriate female, thus resulting in a fully transgenic animal (Wilmut *et al.*, *Nature* 385:810-813, 1997).

In general, expression of any transgene depends upon its integration position and copy number. After a transgenic animal having the appropriate transgene expression level and tissue-specific transgene expression pattern is obtained by traditional methods (*e.g.*, pronuclear injection or generation of chimeric embryos), the animal is bred in order to obtain progeny having the same transgene expression level and pattern. There are several limitations to this approach. First, transmission of the transgene to offspring does not occur in transgenic chimeras lacking transgenic germ cells. Second, because a heterozygous transgenic founder is bred with a non-transgenic animal, only half of the progeny will be transgenic. Third, the number of transgenic progeny is further limited by the length of the gestation period and number of offspring per pregnancy. Finally, the number of useful transgenic progeny may be further limited by gender: for example, only female animals are useful for producing rHuAFP expressed in milk. In view of these limitations, nuclear transfer technology provides the advantage of allowing, within a relatively short time period, the generation of many female transgenic animals that are genetically identical.

Animals expressing rHuAFP in their milk also may be generated by direct transfer of the transgene into the mammary tissue of post-partum animals (Karatzas *et al.*, U.S. patent No. 5,780,009). Such animals do not contain the

transgene within their germline, and hence do not give rise to transgenic progeny.

Screening for Transgenic Animals

After the candidate transgenic animals are generated, they must be screened in order to detect animals whose cells contain and express the transgene. The presence of a transgene in animal tissues is typically detected by Southern blot analysis or by employing PCR-amplification of DNA from candidate transgenic animals (see, *e.g.*, Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1998; see also Lubon *et al.*, U.S.P.N. 5,831,141). rHuAFP expression in milk or urine may be determined by any standard immmunological assay, for example, ELISA or Western blotting analysis, using an anti-AFP antibody (see, *e.g.*, Murgita *et al.*, U.S.P.N. 5,384,250 and Ausubel *et al.*, *supra*). For a working example of ELISA-based detection of transgene-encoded protein in milk, see Drohan *et al.*, U.S.P.N. 5,589,604.

Purification of AFP from Urine or Milk

Recombinant protein may be purified from milk or urine using standard protein purification techniques, such as affinity chromatography (see, *e.g.*, Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1998; see also Lubon *et al.*, U.S.P.N. 5,831,141) or other methods known to those skilled in the art of protein purification. Once isolated, the recombinant protein can, if desired, be further purified by *e.g.*, by high performance liquid chromatography (HPLC; *e.g.*, see Fisher, *Laboratory Techniques In Biochemistry And Molecular Biology*, eds. Work and Burdon, Elsevier, 1980). Preferably, the purification is by at least 2-fold, more preferably, by at least 10-fold, still more preferably, by at least 100-fold, and

most preferably, by at least 1000-fold.

Use of rHuAFP Purified from Milk or Urine of Transgenic Animals

rHuAFP (Murgita *et al.*, U.S.P.N. 5,384,250) in milk or urine or purified from milk or urine may be used as a diagnostic standard (*e.g.*, for detection of increased levels of AFP in adult human serum, which may indicate the presence of cancer or liver regeneration) or as a therapeutic. For example, rHuAFP produced by the methods of the invention may be administered to mammals to inhibit cancer cell growth, to induce bone marrow cell proliferation (for example, after a bone marrow transplant or after administration of a myelotoxic treatment such as chemotherapy), or as an immunosuppressive agent (for example, to treat systemic lupus erythematosus, myasthenia gravis, insulin-dependent diabetes mellitus, or to inhibit rejection of a transplanted organ).

rHuAFP in milk or urine or purified from milk or urine may be administered in an effective amount either alone or in combination with a pharmaceutically acceptable carrier or diluent, either alone or in combination with other therapeutic agents by any convenient means known to skilled artisans, *e.g.*, intravenously, orally, intramuscularly, or intranasally.

Example I: Generation of Transgenic Goats Expressing Recombinant Human AFP (rHuAFP)

Transgene construction and generation of transgenic goats

Transgenic goats expressing rHuAFP in their milk, under the control of the goat beta-casein promoter, are generated as follows. A DNA fragment containing the full length coding region of human AFP and lacking the translational start sequence is obtained by performing polymerase chain reaction (PCR) amplification using a plasmid containing the HuAFP cDNA

(Genbank Accession No. J00077), such as pHuAFP (described in Murgita *et al.*, U.S.P.N. 5,384,250) as a template and the following oligonucleotide primers: NH_2 (5'-AAA CTC GAG AAG TGG GTG GAA-3') and COOH (5-AAA CTC GAG TTA AAC TCC CAA AGC-3').

Each PCR reaction contains 34 µl DNA template, 10 µl of 10 pmol/µl 5'-primer, 10 µl 10X reaction buffer, 20 µl 1mM dNTP's, 2 µl DMSO and 1 µl DNA template, 10 µl of 10 pmol/µl of 10 pmol/µl 5'primer, 10 µl of 10 pmol/µl 3'-primer, 1 µl glycerol, 10 µl DMSO and 1 µl *Pfu* DNA polymerase. Annealing, extension, and denaturation temperatures are 50°C, 72°C and 94°C, respectively, for 30 cycles, using the Gene Amp PCR System 9600. The 1783-bp DNA obtained from the PCR reactions is digested with *Xho* I and then purified by isolating the fragment from a 0.7% TAE agarose gel, followed by gel extraction employing the Geneclean method (Bio 101; Vista, CA) according to the manufacturer's instructions.

The transgene vector (see Figure 1; see Meade *et al.*, U.S.P.N. 5,827,690) contains an altered goat beta-casein gene with an *Xho* I site in place of the coding portion of the gene. The portion deleted from the goat beta-casein gene extends from the *Taq* I site in exon 2 to the *Ppu* MI site in exon 7. Exon 2 contains the translational start codon in addition to a 15 amino acid secretion signal. To generate the goat beta-casein/human AFP transgene, the *Xho* I/*Xho* I HuAFP cDNA is ligated between exons 2 and 7 of the goat beta-casein gene at the *Xho* I site. The complete transgene contains 6.2 kb of 5' goat beta-casein sequence, the 1.8 kb HuAFP cDNA, and the 7.1 kb 3' goat beta-casein flanking sequence.

Transgenic goats are generated by injecting, into the pronucleus of collected embryos, the 15.1 kb fragment of the goat beta-casein-HuAFP purified free from procaryotic DNA at a concentration of 1.0 µg/ml in 10 mM Tris, pH 7.5, 0.1 mM EDTA. Injected embryos are then transferred to recipient

females. A founder (F_0) transgenic goat is identified by analyzing genomic DNA from blood by polymerase chain reaction (PCR) and by Southern blot analysis in order to detect the presence of the transgene. For PCR analysis, the same two oligonucleotides that are employed to generate the HuAFP cDNA are used in the reaction. For Southern blot analysis, the DNA is fractionated on a 1% TBE agarose gel, blotted onto nitrocellulose, and probed with a random-primed ^{32}P-labelled 1.8 kb HuAFP cDNA. The identified founder is then bred to a nontransgenic animal to produce transgenic offspring. Alternatively, transgenic offspring may be obtained by nuclear transfer, as described above. Transmission of the transgene is detected by analyzing genomic DNA from blood as described above.

Lactation induction

Female animals twelve months of age or older are induced to lactate by hormone therapy and hand stimulation over a 12 day period. During the first 4 days, the animal receive subcutaneous injections of 0.1 mg/kg of estradiol 17-β and 0.25 mg/kg of progesterone dissolved in 100% ethanol. This daily amount is divided between morning and evening injections. The udder is palpated once daily and the teats are hand-stimulated for 5-10 minutes each morning. Lactating transgenic females are milked manually twice per day and the milk is stored frozen at -20°C.

Protein purification

Transgenic goat milk containing rHuAFP is thawed and the pH adjusted to 4.4 with glacial acetic acid to precipitate out the casein. The resultant precipitate is removed by centrifugation at 8000 x g for 20 min. at 4°C. The supernant is adjusted to pH 5.5 with NaOH and filtered through a 22 µm filter. The rHuAFP is purified from the whey fraction by applying the

filtrate to a Butyl-Toyopearl column which is equilibrated in 0.2 M sodium phosphate, 0.1 M arginine-HCl, 0.01% Tween 80, pH 6.0. The rHuAFP is eluted with a solution of 0.2 M sodium phosphate, 0.1 M arginine-HCl, 70% ethylene glycol. Fractions containing rHuAFP, determined by Western blot or ELISA, are pooled and dialyzed against 30 mM Tris-HCl, pH 8.0. Final purification of rHuAFP is achieved by applying the dialyzed sample onto a Mono Q column equilibrated in 20 mM Tris-HCl, pH 8.0. Bound proteins are eluted during a step gradient from 0-100% 91 M NaCl, 20 mM Tris-HCl, pH 8.0).

Example II: Design of a Genomic Alpha Fetoprotein Transgene Expression Construct

Human AFP Gene Cloning

The gene for human AFP spans roughly 19 kb and contains 15 exons (14 coding) separated by 14 introns. The complete sequence of the human AFP gene has been reported by Gibbs *et al.* (*Biochemistry* 26:1332-1343, 1987) and set forth in GenBank Accession No. M16610. The gene was initially cloned in two fragments of approximately 15 kb, which were then combined, to generate the expressed protein.

A human placental genomic library (Stratagene, La Jolla, CA), with an average insert size of between 9 and 23 kb, was initially screened with a series of complementary oligonucleotide probes which recognize exons at the beginning, middle, and end of the human AFP gene. The first screen did not produce any positive clones. Two larger DNA probes were then made by using the polymerase chain reaction (PCR) to amplify regions of the beginning and end of the AFP gene from human genomic DNA (arrows, Figure 2). Subsequent screening of the library with these probes produced two overlapping lambda (λ) phage clones, of approximately 15 kb, which together

span the length of the human AFP gene (Figure 2).

Construct Design

The two phage inserts were then subcloned into a superCOS 1 vector (this vector was used because it can accommodate larger DNA inserts). The two resulting subclones, gtc912 and gtc913 are then manipulated, as follows, to generate the final expression constructs. First, sequences 5' and 3' of the coding region are removed. In addition, at the 5' end, a Kozak sequence is added to ensure efficient initiation of translation. This is accomplished by inserting restriction enzyme "linkers" into the gene sequences for the subsequent excision of the appropriate sequences, leaving the flanking sequences intact (Figures 3&4). Second, the 5' and 3' pieces are excised from their respective vectors using an enzyme common to the two inserts which allows them to be joined together to form the complete gene. The enzyme BglI, is used since it cuts once at the 3' end of the 5' piece (IK179) and once, at the same site, at the 5' end of the 3' piece (IK175). Finally, these two pieces are linked together in a superCos plasmid vector in the SalI site and then the entire genomic fragment is placed into the SalI site of a GTC beta-casein expression vector (Figure 5).

The genomic AFP gene construct, if desired, may be mutated at its single N-linked glycosylation site. Using restriction sites flanking the glycosylation site (*e.g.*, DsaI and BlpI), an oligonucleotide containing the mutation (N to Q) can be substituted using standard molecular biological techniques (*e.g.*, gapped mutagenesis). The non-glycosylated version of the genomic AFP is then ligated into the beta-casein vector as described above and used to generate a transgenic animal, *e.g.*, a mouse, goat, sheep, pig, or cow.

The publications listed hereafter describe the generation, detection, and analysis of transgenic animals that secrete recombinant proteins into milk, as well as purification of the recombinant proteins. These publications are

herein incorporated by reference: Hurwitz *et al.*, U.S.P.N. 5,648,243 (goats); Meade, *et al.*, U.S.P.N. 5,827,690 (goats); DiTullio *et al.*, U.S.P.N. 5,843,705 (goats); Clark *et al*, U.S.P.N. 5,322,775 (sheep); Garner *et al.*, U.S.P.N. 5,639,940 (sheep); Deboer *et al.*, U.S.P.N. 5,633,076 (cows); and Drohan *et al.*, U.S.P.N. 5,589,604 (pigs and mice). Kerr *et al.*, *Nat. Biotechnol.* 16:75-79, 1998, herein incorporated by reference, describes the generation and analysis of transgenic animals that excrete recombinant proteins into urine, as well as purification of the recombinant proteins.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure that come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth.

What is claimed is:

CLAIMS

1. A substantially pure nucleic acid molecule comprising: (i) a nucleic acid sequence encoding recombinant human alpha-fetoprotein (rHuAFP), (ii) a milk-specific promoter, said promoter being operably linked to said rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of said rHuAFP by milk-producing cells into the milk of a mammal.

2. A substantially pure nucleic acid molecule comprising: (i) a nucleic acid sequence encoding recombinant human alpha-fetoprotein (rHuAFP), (ii) a urine-specific promoter, said promoter being operably linked to said rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of said rHuAFP by urine-producing cells into the urine of a mammal.

3. A non-human transgenic mammal that expresses recombinant human alpha-fetoprotein (rHuAFP) in its milk, wherein milk-producing cells of said mammal contain a transgene that comprises: (i) a nucleic acid sequence encoding rHuAFP, (ii) a milk-specific promoter, said promoter being operably linked to said rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of said rHuAFP by milk-producing cells into the milk of a mammal.

4. A non-human transgenic mammal that expresses recombinant human alpha-fetoprotein (rHuAFP) in its urine, wherein urine-producing cells of said mammal contain a transgene that comprises: (i) a nucleic acid sequence encoding rHuAFP, (ii) a urine-specific promoter, said promoter being operably

linked to said rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of said rHuAFP by urine-producing cells into the urine of an animal.

5. The non-human transgenic mammal of claim 1 or 2, wherein the mammal is a goat, a cow, a sheep, or a pig.

6. Non-human mammal's milk comprising recombinant human alpha-fetoprotein (rHuAFP).

7. The milk of claim 6, wherein the rHuAFP is soluble and is produced by a non-human transgenic mammal whose milk-producing cells express a transgene that comprises: (i) a nucleic acid sequence encoding rHuAFP, (ii) a milk-specific promoter, said promoter being operably linked to said rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of said rHuAFP by said milk-producing cells into the milk of said mammal.

8. Non-human mammal's urine comprising recombinant human alpha-fetoprotein (rHuAFP).

9. The urine of claim 8, wherein the rHuAFP is soluble and is produced by a non-human transgenic mammal whose urine-producing cells express a transgene that comprises: (i) a nucleic acid sequence encoding rHuAFP, (ii) a urine-specific promoter, said promoter being operably linked to said rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of said rHuAFP by said urine-producing cells into the urine of said mammal.

10. A method of producing recombinant human alpha-fetoprotein (rHuAFP) that is secreted in the milk of a mammal, said method comprising the steps of:

(a) providing a cell transfected with a transgene that comprises: (i) a nucleic acid sequence encoding rHuAFP, (ii) a milk-specific promoter, said promoter being operably linked to said rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of said rHuAFP by a milk-producing cell, wherein said milk-producing cell is derived from said transfected cell;

(b) growing said cell to produce a mammal comprising milk-producing cells that express and secrete said rHuAFP into said milk; and

(c) collecting said milk containing said rHuAFP from said mammal.

11. The method of claim 10, wherein said rHuAFP is purified from said milk.

12. A method of producing recombinant human alpha-fetoprotein (rHuAFP) that is secreted in the urine of a mammal, said method comprising the steps of:

(a) providing a cell transfected with a transgene that comprises: (i) a nucleic acid sequence encoding rHuAFP, (ii) a urine-specific promoter, said promoter being operably linked to said rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of said rHuAFP by a urine-producing cell, wherein said urine-producing cell is derived from said transfected cell;

(b) growing said cell to produce a mammal comprising urine-producing cells that express and secrete said rHuAFP into said urine; and

(c) collecting said urine containing said rHuAFP from said mammal.

13. The method of claim 12, wherein said rHuAFP is purified from said urine.

14. A method of treating a patient in need of recombinant human alpha-fetoprotein (rHuAFP), said method comprising administering to said patient a therapeutically-effective amount of non-human mammal's milk comprising recombinant human alpha-fetoprotein (rHuAFP).

15. The method of claim 14, wherein said rHuAFP is produced by a non-human transgenic mammal whose milk-producing cells contain a transgene that comprises: (i) a nucleic acid sequence encoding rHuAFP, (ii) a milk-specific promoter, said promoter being operably linked to said rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of said rHuAFP by milk-producing cells into the milk of said mammal.

16. A method of treating a patient in need of recombinant human alpha-fetoprotein (rHuAFP), said method comprising administering to said patient a therapeutically-effective amount of recombinant human alpha-fetoprotein (rHuAFP) purified from a non-human mammal's urine.

17. The method of claim 16, wherein said rHuAFP is produced by a non-human transgenic mammal whose urine-producing cells contain a transgene that comprises: (i) a nucleic acid sequence encoding rHuAFP, (ii) a urine-specific promoter, said promoter being operably linked to said rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of said rHuAFP by urine-producing cells into the urine of said mammal.

18. The method of claim 14 or 16, wherein said method is for the treatment of cancer.

19. The method of claim 14 or 16, wherein said method is for suppressing the immune system of a patient in need thereof.

20. The method of claim 14 or 16, wherein said method is for inducing proliferation of bone marrow cells in a patient in need thereof.

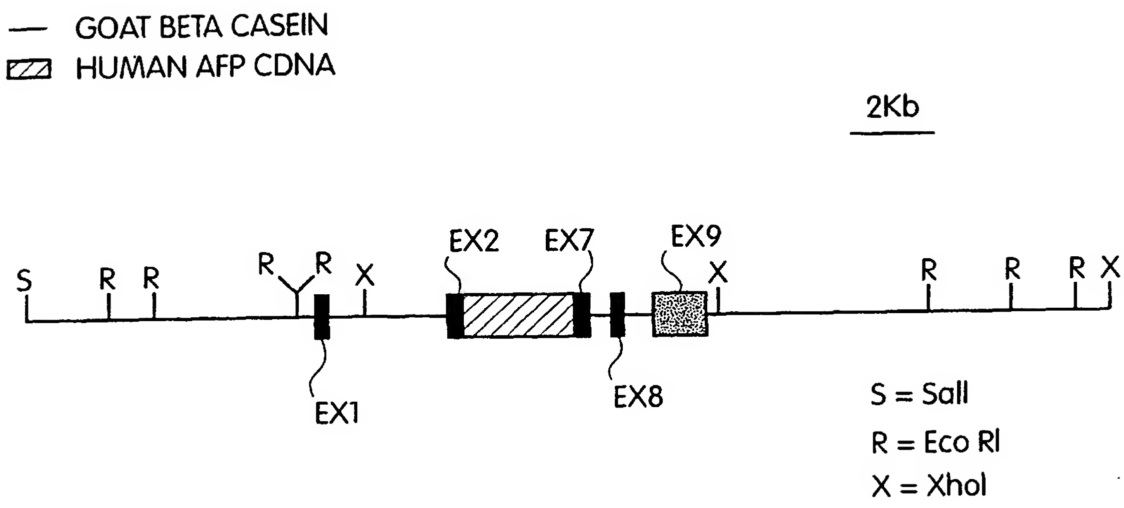

Fig. 1

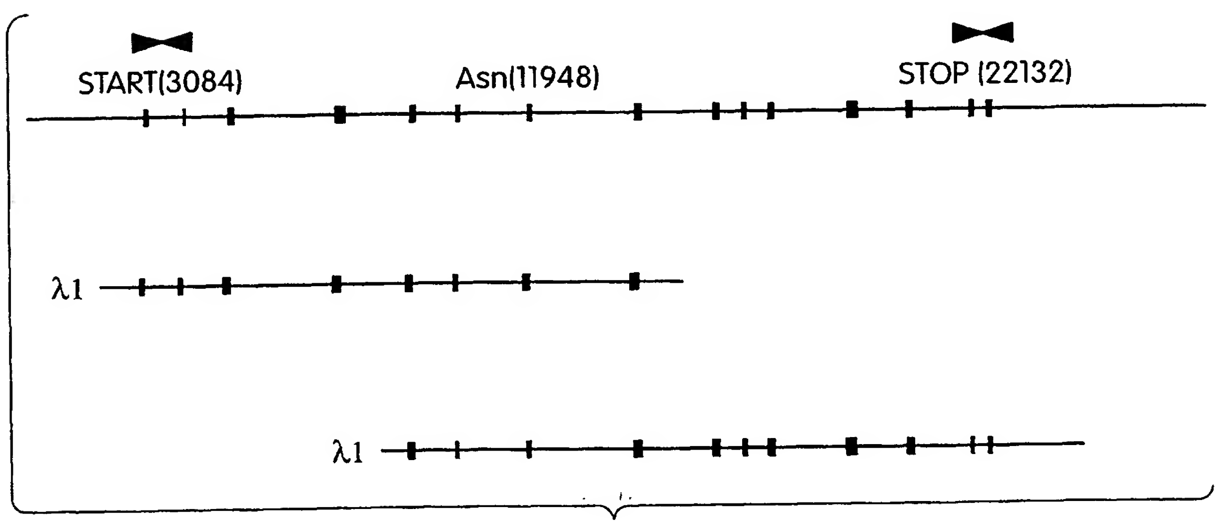

Fig. 2

RECTIFIED SHEET (RULE 91)

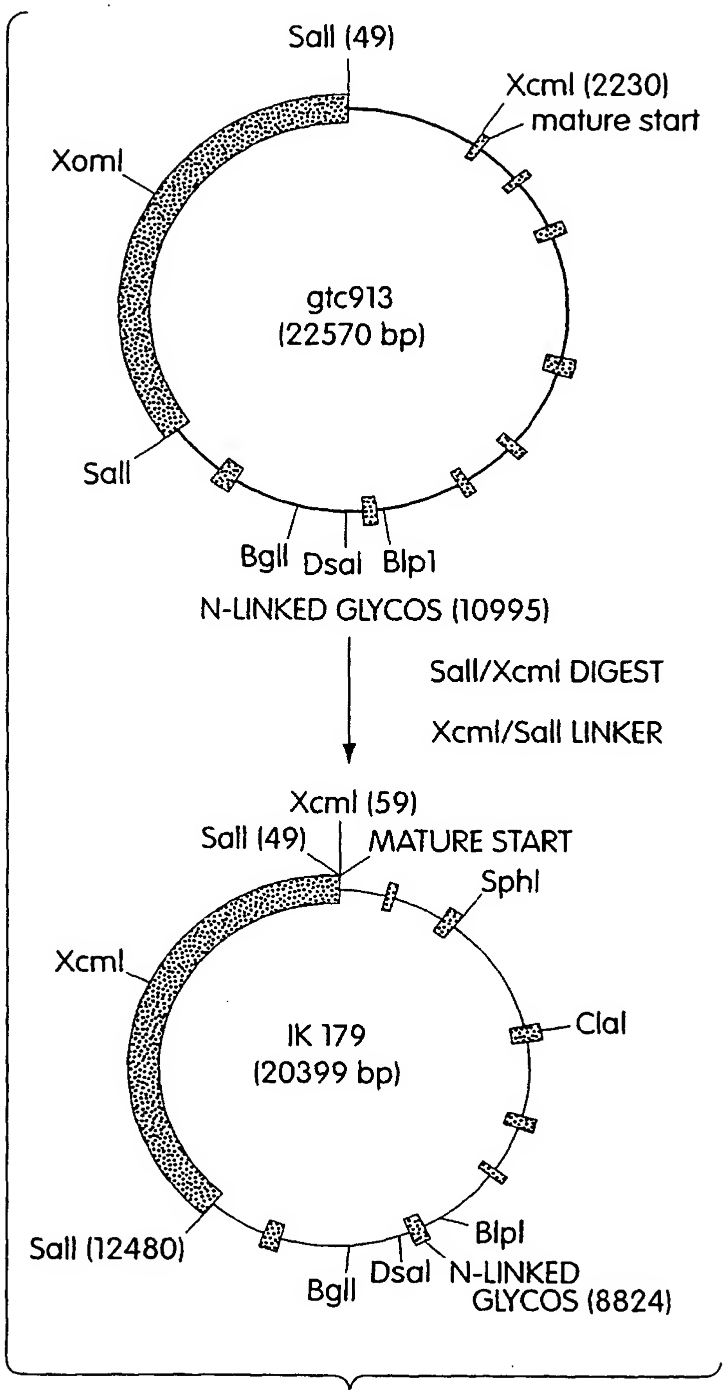
SalI (49)
XcmI (2230)
mature start
XomI
gtc913
(22570 bp)
SalI
BglI
DsaI
Blp1
N-LINKED GLYCOS (10995)
SalI/XcmI DIGEST
XcmI/SalI LINKER
XcmI (59)
SalI (49)
MATURE START
SphI
XcmI
ClaI
IK 179
(20399 bp)
SalI (12480)
BlpI
BglI
DsaI
N-LINKED
GLYCOS (8824)

Fig. 3

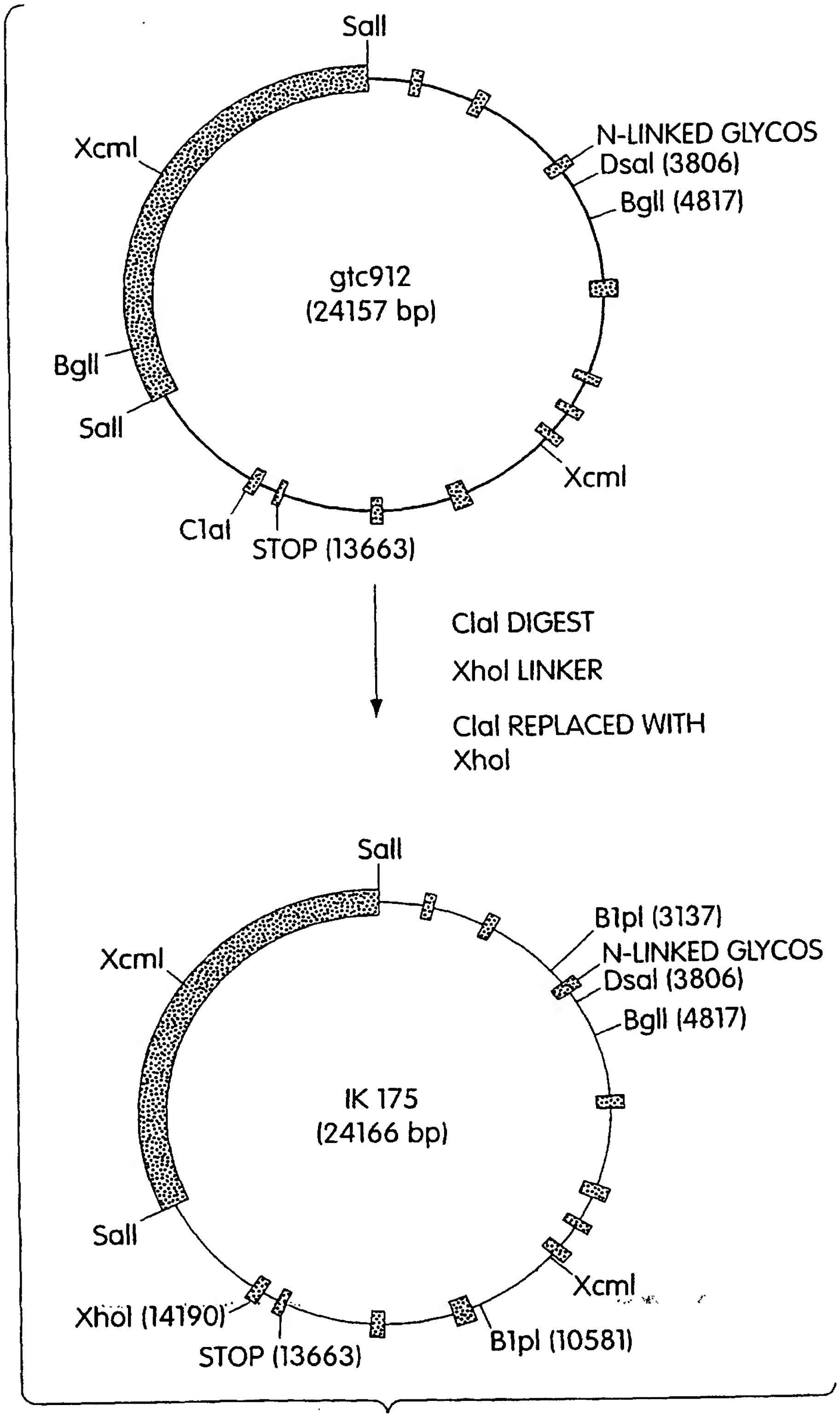
SalI
XcmI
N-LINKED GLYCOS
DsaI (3806)
BglI (4817)
gtc912
(24157 bp)
BglI
SalI
XcmI
ClaI
STOP (13663)
ClaI DIGEST
XhoI LINKER
ClaI REPLACED WITH
XhoI
SalI
XcmI
B1pI (3137)
N-LINKED GLYCOS
DsaI (3806)
BglI (4817)
IK 175
(24166 bp)
SalI
XcmI
XhoI (14190)
STOP (13663)
B1pI (10581)

Fig. 4

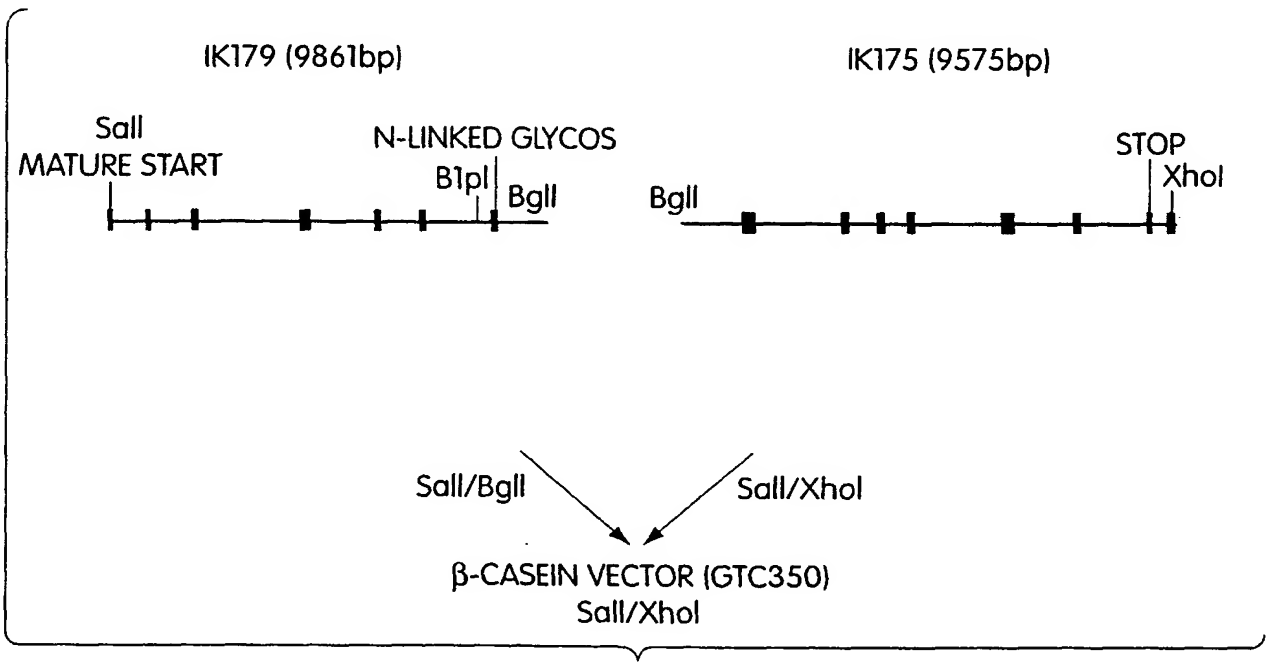
IK179 (9861bp)
IK175 (9575bp)
SalI
MATURE START
N-LINKED GLYCOS
B1pI
BglI
BglI
STOP
XhoI
SalI/BglI
SalI/XhoI
β-CASEIN VECTOR (GTC350)
SalI/XhoI

Fig. 5

PCT
WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A01K 67/00, 67/033, 67/027, C12N 5/00, 5/02, C07K 1/00, A61K 39/00

A3

(11) International Publication Number: WO 00/40693

(43) International Publication Date: 13 July 2000 (13.07.00)

(21) International Application Number: PCT/US00/00264

(22) International Filing Date: 6 January 2000 (06.01.00)

(30) Priority Data:
60/114,995 6 January 1999 (06.01.99) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/114,995 (CIP)
Filed on 6 January 1999 (06.01.99)

(71) Applicant (*for all designated States except US*): ATLANTIC BIOPHARMACEUTICALS, INC. [US/US]; 50 Church Street, Cambridge, MA 02138 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): LINDSAY, Stace [US/US]; 8 Cypress Street, Cambridge, MA 02140 (US). MULROY, Robert [US/US]; 19 Hilliard Street, Cambridge, MA 02138 (US). SEMENIUK, Daniel [US/US]; 1860 Thousand Oaks Lane, Lawrenceville, GA 30043 (US).

(74) Agent: CLARK, Paul, T.; Clark & Elbing LLP, 176 Federal Street, Boston, MA 02110-2214 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(88) Date of publication of the international search report: 28 September 2000 (28.09.00)

(54) Title: EXPRESSION OF SECRETED HUMAN ALPHA-FETOPROTEIN IN TRANSGENIC ANIMALS

(57) Abstract

The invention features a process of expressing secreted recombinant human alpha-fetoprotein (rHuAFP) in the milk or urine of transgenic mammals.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/00264

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :Please See Extra Sheet.
US CL : 800/3, 14; 435/325; 530/350; 424/277.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/3, 14; 435/325; 530/350; 424/277.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, SCISEARCH, CAPLUS, WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BENNETT et al. Similarity Between Natural and Recombinant Human Alpha-Fetoprotein as Inhibitors of Estrogen-Dependent Breast Cancer Growth. Breast Cancer Research and Treatment. September 1992. Vol. 45. No. 2. pages 169-179, especially page 175.	14,16, 18-19
Y	KOYAMA et al. Lectin Affinity Electrophoretic Demonstration of Tissue Specificity and Malignant Alteration of Human a-Fetoprotein Isoforms Produced in Transgenic Mice. Biochemical and Biophysical Research Communications. June 1996. Vol. 223. No. 3. pages 757-761, especially page 758.	14,16, 18-19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date		
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 MARCH 2000	03 JUL 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Jerry J Dey for PETER PARAS JR Telephone No. (703) 308-8340

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/00264

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DUDICH et al. Growth-Regulative Activity of Human Alpha-Fetoprotein for Different Types of Tumor and Normal Cells. Tumor Biology. January-February 1998. Vol. 19. No. 1. pages 30-40, especially page 36.	14,16, 18-20
A	Database SCISEARCH, Accession No. 1999:701362, BIRRER et al. 'The Immunology of Alphafetoprotein.' Abstract, Journal of Tumor Marker Oncology. Fall 1999. Vol. 14. No. 3. pages 55-62, see entire document.	1-20
Y, E	US 6,013,857 A (DEBOER et al) 11 January 2000 (11.01.00), see columns 6-7.	1-3,5-7, 10-11, 14-15
Y	WO 96/22787 A1 (MURGITA et al) 01 August 1996(01.08.96), especially pages 1-4.	14,16, 18-20
Y	US 5,766,884 A (TOWNES et al) 16 June 1998(16.06.98), see column 15, lines 5-55.	1-17
Y	US 5,843,776 A (TAMAOKI et al) 01 December 1998(01.12.98), see columns 5-6.	1-17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/00264

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

A01K 67/00, 67/033, 67/027; C12N 5/00, 5/02; C07K 1/00; A61K 39/00